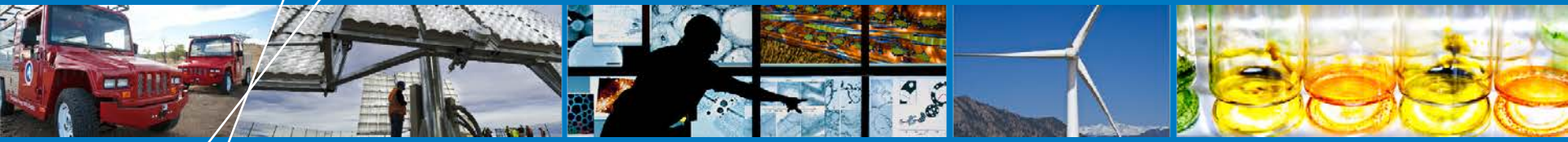
NREL
NATIONAL RENEWABLE ENERGY LABORATORY

Biological Systems for Hydrogen Photoproduction



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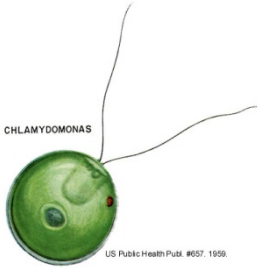
Overview

Timeline

- Project start date: FY00
- Project end date: 9/30/2013*
- Percent complete: 85%

*Project continuation and direction determined annually by DOE

Barriers



- Barriers addressed:
  - Rate of H_2 production (AO)
  - Oxygen Accumulation (AP)
- Targets:
  - Duration of production
  - Solar to H_2 (STH) energy conversion

Budget

- Total project funding: $10,551K
- Funding received in FY12: $600K
- Planned funding for FY13: $480K

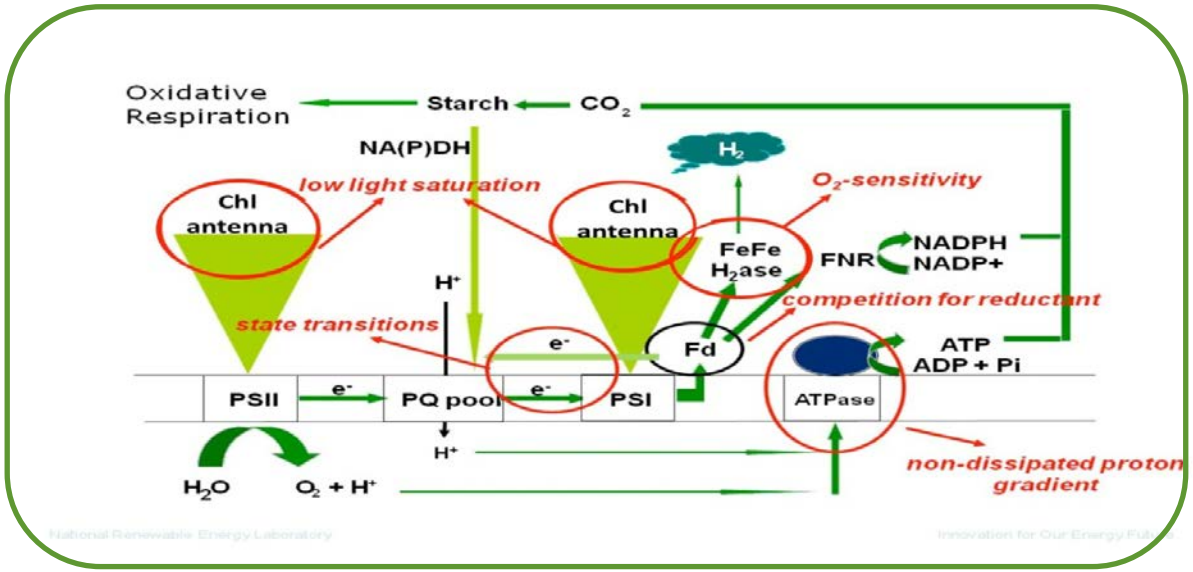
Partners (in FY12)

- Dr. Sergey Kosourov, Institute of Basic Biological Problems, RAS, Pushchino, Russia
- Dr. Eric Johnson, Johns Hopkins University

Relevance

Photobiological water splitting coupled to hydrogenase-mediated H_2 production has the potential to convert *about* ***10% of incident solar energy*** *into* H_2. Various barriers have been identified as currently limiting green algal H_2 production, including:

- the O_2 sensitivity of the hydrogenase enzyme,
- the competition for reductant with CO_2 fixation and cyclic electron flow,
- the down-regulation of photosynthesis due to non-dissipation of the proton gradient and state transitions, and
- the low light-saturation of photosynthesis.



Objectives

- **General goal:** Develop photobiological systems for large-scale, low cost and efficient H_2 production from water (barriers AO and AP).

Technical Targets: Photolytic Biological Hydrogen Production					
Characteristics	**Units**	**2012 Status**	**2015 Target**	**2020 Target**	**Ultimate Target**
Duration of continuous H_2 production at full sunlight intensity	**Time units**	**N/A**	**30 min**	**4 h**	**8 h**
Solar to H_2 (STH) energy conversion ratio	**%**	**N/A**	**2**	**5**	**17**

- **Specific tasks:**

  Task 1: Address the O_2 sensitivity of hydrogenases that prevent continuity of H_2 photoproduction under aerobic, high solar-to-hydrogen (STH) conversion efficiency conditions.

  Task 2: Utilize a limited STH H_2-producing method (sulfur deprivation) as a platform to address or test other factors limiting commercial algal H_2 photoproduction, including low rates due to biochemical and engineering mechanisms – ***discontinued in FY13 due to budget restrictions***

Approach/Milestones – Task 1

Task 1: Address the O_2 sensitivity of hydrogenase by introducing the gene encoding for a hydrogenase from *Clostridium acetobutylicum* that is more O_2-tolerant *in vitro* into the photosynthetic alga *Chlamydomonas reinhardtii*; measure its linkage to water oxidation and *in vivo* O_2 tolerance.

Dr. Paul King, NREL

Seth Noone, NREL

Task 1	FY12 Milestone	Due date	Status
3.3.5	Demonstrate expression of an active Ca1 in a *C. reinhardtii* hydrogenase-less background and characterize O_2-sensitivity of light-driven H_2 production (PsaD-Ca1 construct)	9/30/12	Completed

	FY13 Milestones	Due date	Status
3.3.1-1	Re-test the H_2 photoproduction activity of the PsaD-Ca1 transformant strain 55 by the Clark electrode	12/12	Completed
3.3.1-2	Screen 300 HYDA-Ca1transformants for the presence of the Ca1 gene and test positive transformants for H_2 production activity through the plate assay and Clark electrode	2/13	Completed
3.3.1-3	Generate new transformants with 3 variations of added introns from, respectively, RbcS2, HYDA1 and HYDA2	4/13	On track
3.3.1-4	Test at least 100 strains from the first generation of intron-containing transformants for H_2-production activity through the plate assay (contingent upon finding a new post-doc to perform the work)	5/13	On track
3.3.1-5	Go/NoGo: if addition of introns increases the H_2 photoproduction activity and stability of Ca1 by at least 3 times compared to HYDA1 of PsaD-based constructs, use the strain for further improvements; if not, propose a new plan for DOE's approval to re-direct the work (*CPS Agreement Milestone*)	7/13	On track

Accomplishments and Progress – Task 1

Task 1 – Expression of the more O_2-tolerant clostridial Ca1 hydrogenase in *C. reinhardtii*

Last year's Progress: A double hydrogenase knock-out mutant (⁻hydA1/hydA2) was isolated under BES funding and served as a host for expression of CaI hydrogenase gene behind the PsaD promoter (which requires light for optimal anaerobic induction).

psad prom	Fd TP	codon-optimized Ca1 (Geneart)	psad term

Construct 1: psad promoter and terminator (constitutive expression) with ferredoxin transit peptide/Ca1 optimized by geneart.

hyda prom	Fd TP	codon-optimized Ca1 (Geneart)	hyda term

Construct 2: native algal hydrogenase promoter and terminator with ferredoxin transit peptide/Ca1 optimized by geneart.

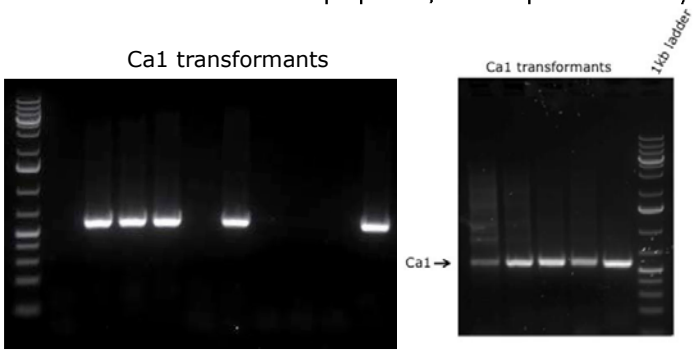
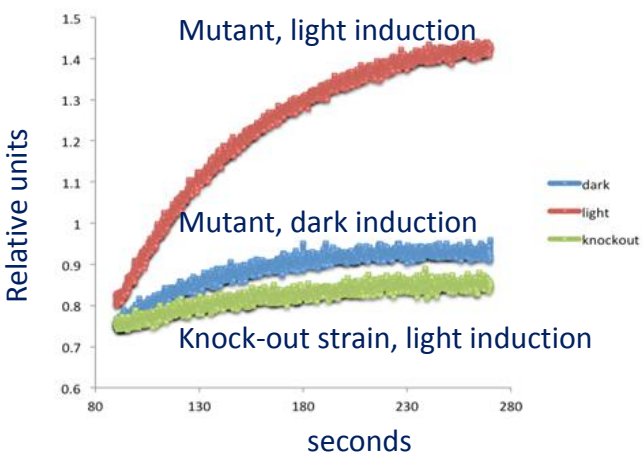


Figure 1. PCR of genomic DNA from various Ca1transformants

PCR (genomic DNA) RT-PCR (mRNA)

The clostridial hydrogenase was inserted into the Chlamydomonas genome and transcribed.

Light-dependent H_2 production was detected!



Accomplishments and Progress - Task 1

Task 1 – Expression of the more O_2-tolerant clostridial Ca1 hydrogenase in *C. reinhardtii* and measurement of its O_2 tolerance - *completed FY12 milestone 3.3.5.*

Strain	Rate of H_2 production in Clark electrode (μmol H_2 mg Chl^{-1} h^{-1})	% WT
WT	260	100
Double knock-out	0.09	0.035
Double knock-out/Ca1	5.1	2

Strain 55 expressing Ca1 photoproduces H_2 at around 2% of WT rate

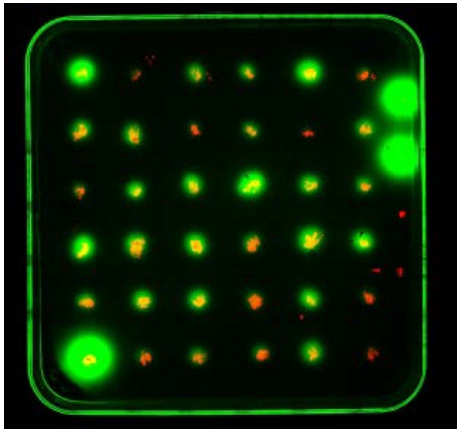
Ca1 activity is not lost upon subsequent cycles of illumination, suggesting higher tolerance to photosynthetically-produced O_2

Strain	Cycle of illumination	Rate of H_2 production in Clark electrode (μmol H_2 mg Chl^{-1} h^{-1})	Change in rate from 1st light cycle
WT	1	269	N/A
	2	205	-23%
	3	179	-33%
Double knock-out/Ca1	1	5.1	N/A
	2	5.6	+10%
	3	6.2	+25%

Accomplishments and Progress - Task 1

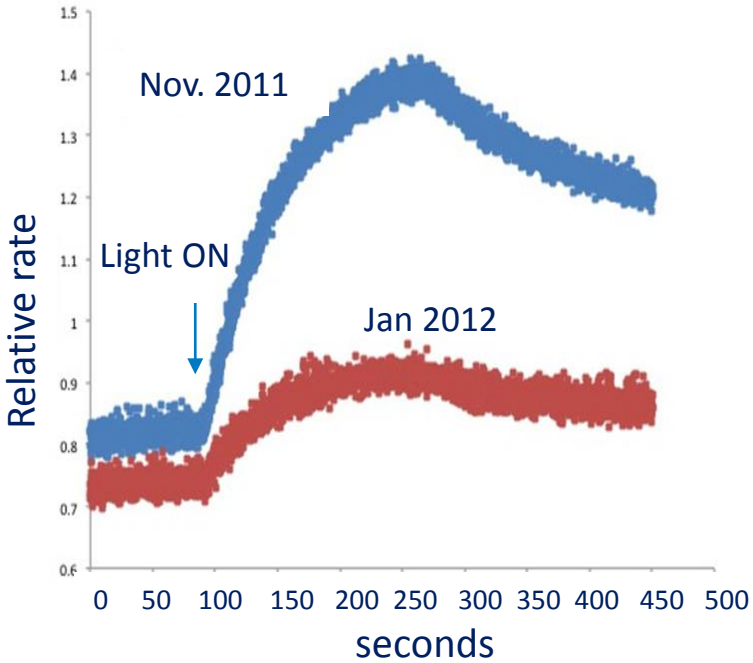
Task 1 – Expression of the more O_2-tolerant clostridial Ca1 hydrogenase in *C. reinhardtii* and measurement of its O_2 tolerance

However, GFP plate screen shows considerable heterogeneity of H_2 production levels among cells grown from a liquid culture of the Ca1-expressing transformant ...



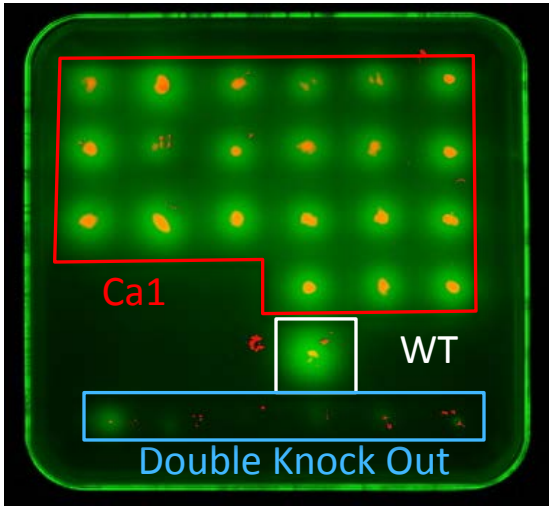
PsaD-Ca1 Strain 55

... and activity is not stable over time if culture is serially propagated.



Accomplishments and Progress – Task 1

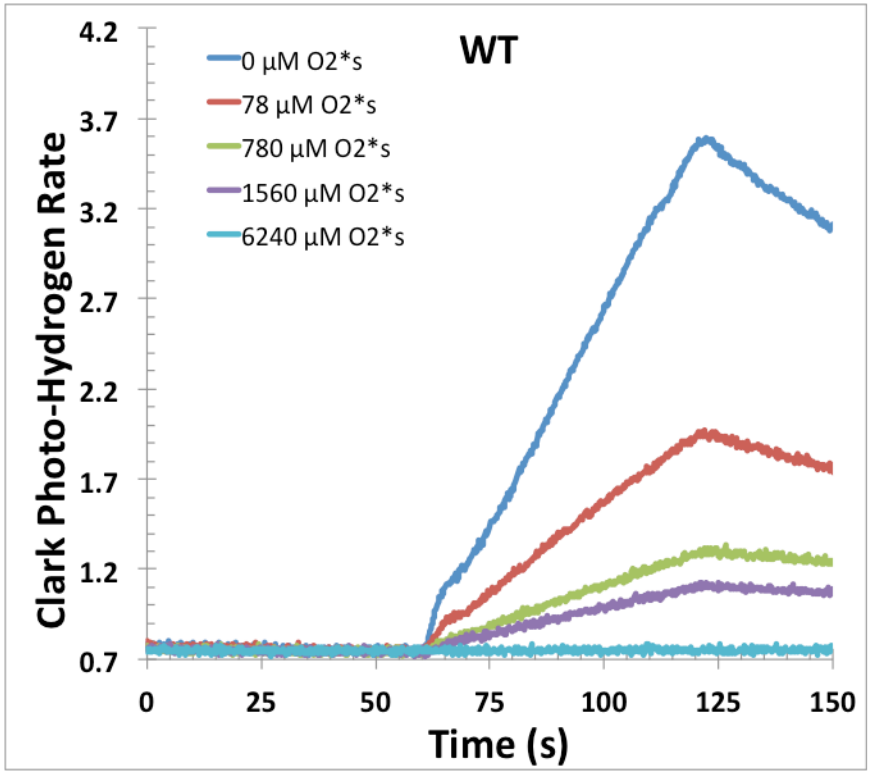
Task 1. Recover original high H_2-producing strain from plates and re-test it – *completion of milestone 3.3.1-1*

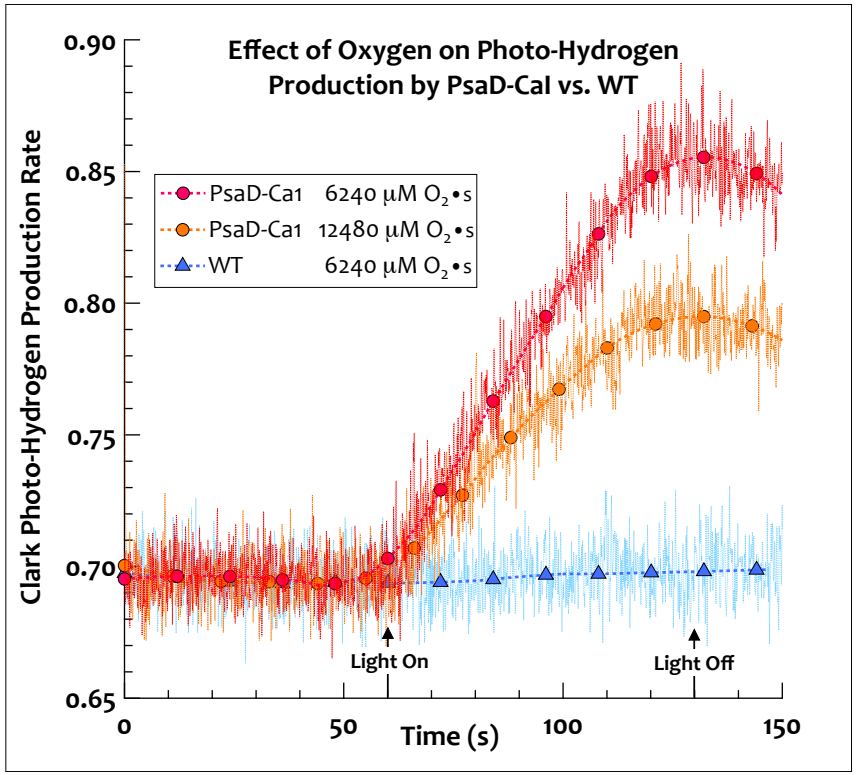


The strain shown as having the highest H_2 production in the previous slide, strain 55 was re-plated, retested by GFP, and showed more uniform H_2 production as shown above.

Accomplishments and Progress – Task 1

Task 1. Recover original high H_2-producing PsaD-CaI strain from plates and re-test – *completion of milestone 3.3.1-1*

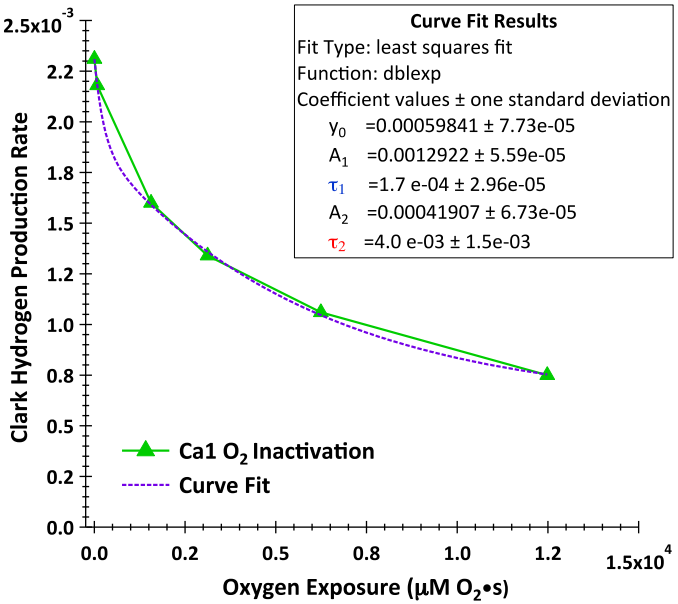


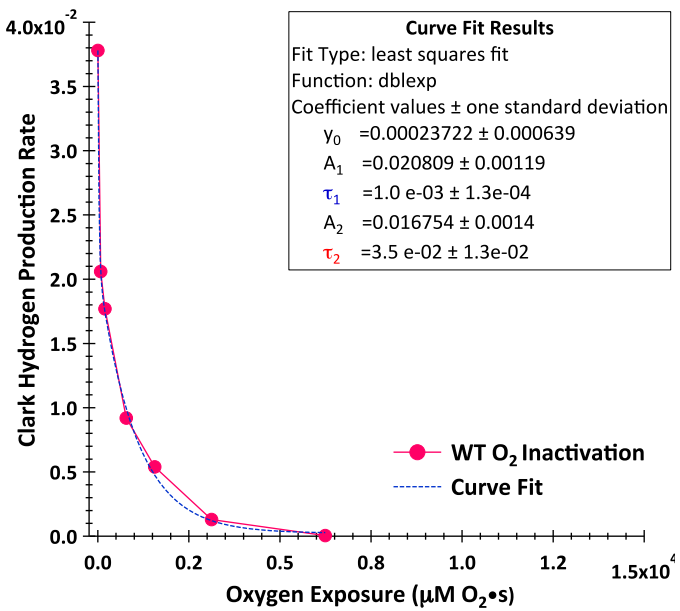


Light-induced H_2 photoproduction by WT and the PsaD-Ca1 transformant were measured with a Clark electrode, following a 1-min dark incubation in the presence of different amounts of added O_2. Relative rates are shown above.

Equivalent to exposure to atmospheric O_2 concentration = 15,600 µM O_2•s

Accomplishments and Progress – Task 1





Clark H_2-Production Rate versus µM O_2•s were plotted, and the results fitted to a bi-exponential-decay curve, according to the equation below. The two inactivation rate constants, τ_1 and τ_2, were estimated for WT and Ca1 cells.

$$y = y_0 + A_1 e^{-x\tau_1} + A_2 e^{-x\tau_2} \qquad x = \frac{(260\ \mu\mathrm{M})(vol_{O_2\ buffer})}{vol_{total}}(Incubation\ time, s)$$

Enzyme/ Strain	O_2 Inactivation Rate Constants (τ_1 and τ_2) (µM O_2^{-1}•s^{-1})		O_2 Tolerance (Ratio to WT)	
	Fast	Slow	Fast	Slow
WT	$2.7 \times 10^{-2} \pm 2 \times 10^{-3}$	$1.7 \times 10^{-3} \pm 5.2 \times 10^{-4}$	NA	NA
PsaDP-Ca1	1.4×10^{-3}	$1 \times 10^{-4} \pm 2 \times 10^{-5}$	19	17
HydAP-Ca1	3×10^{-3}	3.5×10^{-4}	9	5

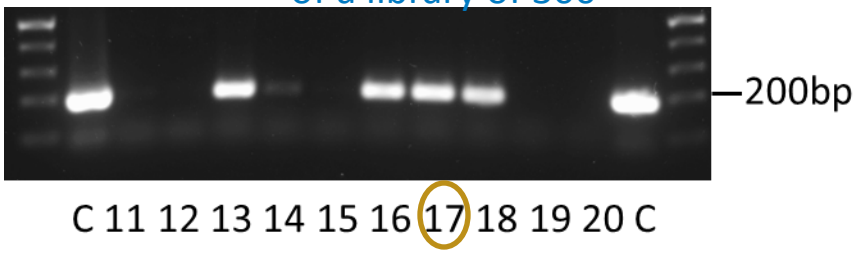
Conclusions:

- *In vivo* expression of Ca1 in *Chlamydomonas* shows higher O_2 tolerance (5-19 fold) for photo-H_2 production than wild-type;
- Level of increased O_2 tolerance of Ca1 compared to HYDA1 *in vivo* is similar to differences of the purified enzymes measured *in vitro*.

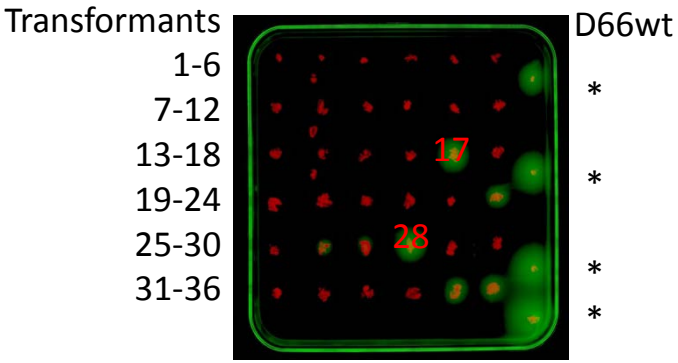
Accomplishments and Progress - Task 1

Task 1. Screen 300 HYDA-Ca1transformants for the presence of the Ca1 gene and test positive transformants for H_2 production activity through the plate assay and Clark electrode – *completion of milestone 3.3.1-2*

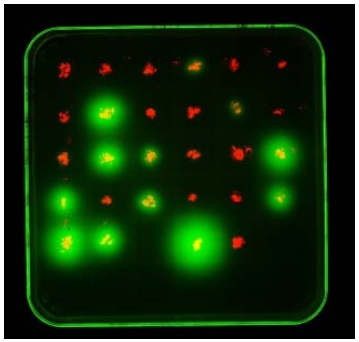
High-throughput PCR screen identified additional transformants carrying the Ca1 gene out of a library of 300



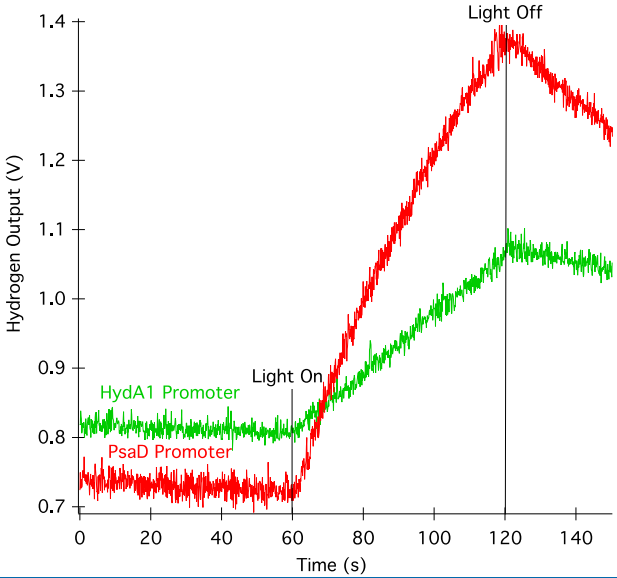
Plating and GFP screening showed two H_2 producing transformants (17, 28)



Strain 28 showed heterogeneity (left) in GFP H_2 plate assay, and photoproduced H_2 at lower rates (right) compared to strains 55



Strain 28



Proposed Future Work

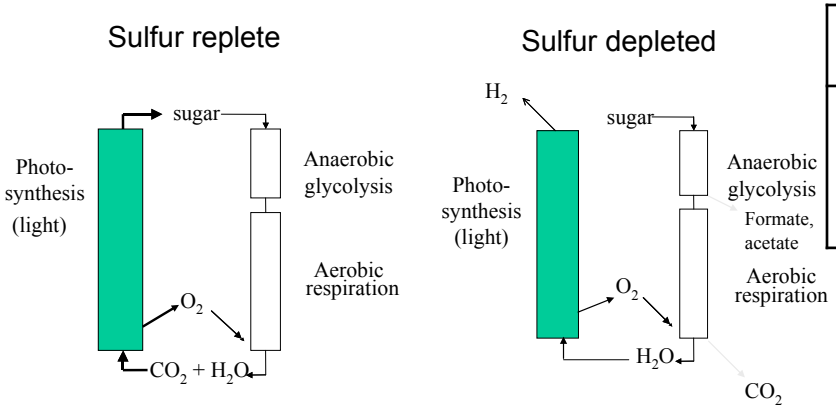
2013

Task 1 – Generate additional Ca1 expression constructs that contain Rubisco Small Subunit or HydA1 introns and test stability and levels of Ca1 activity by GFP plate assay and Clark Electrode; complete Go/NoGo milestone demonstrating at least 3X higher activity and stability of recombinant Ca1 expressed in the *C. reinhardtii* double knock-out background and characterize O_2 sensitivity of light-driven H_2 production.

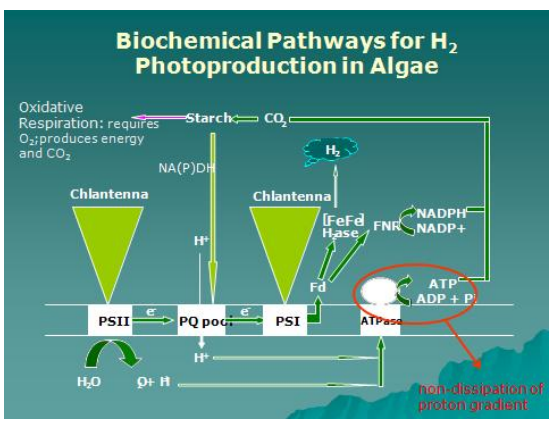
Work beyond FY13: Start to genetically combine selected traits into a strain expressing the Ca1 hydrogenase. Develop photobioreactor systems for cyclic or continuous H_2 production based on current optimization of gas space composition (shown last year) and alginate immobilization.

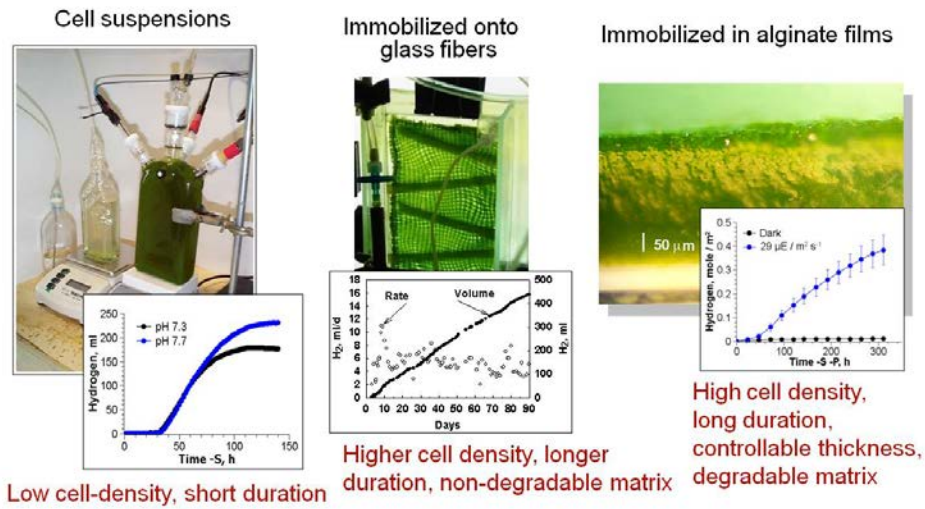
Approach/Milestones – Task 2

Task 2: Utilize the limited STH sulfur-deprivation method to test (a) the rates of H_2 production by inducible ATP synthase mutants that are not limited by the non-dissipation of a proton gradient; and (b) the long-term performance of immobilized algal cultures. *Discontinued in FY13*



Task 2	FY12 Milestones	Due date	Status
3.3.3	Demonstrate continuous operation of the sulfur-deprivation process for a total of 2 months upon addition of phosphate/sulfate to alginate-immobilized cultures.	9/30/12	Completed

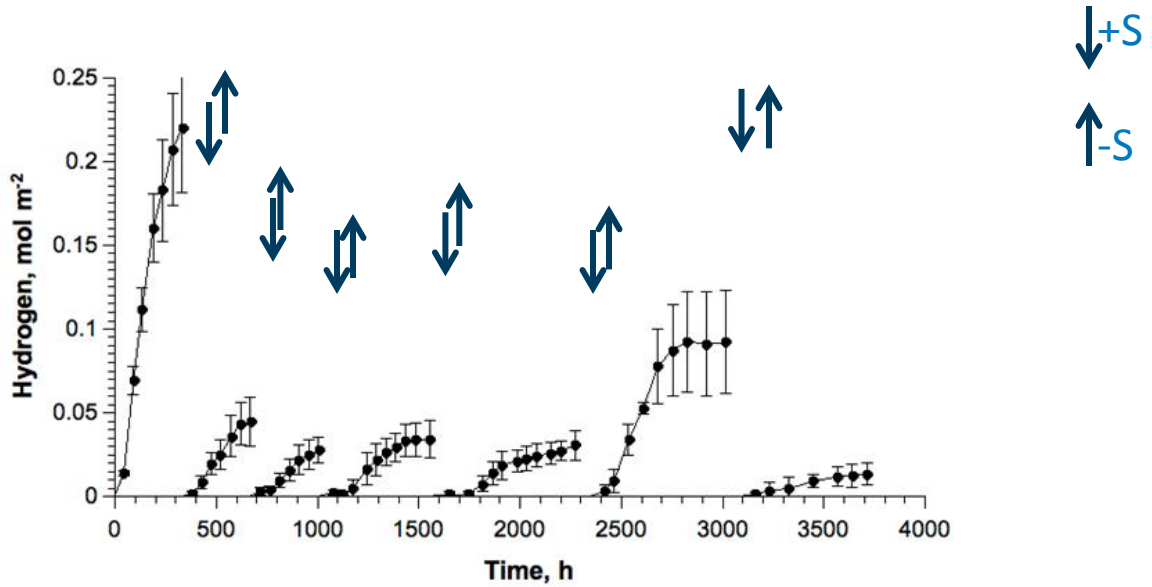




Accomplishments and Progress – Task 2

Task 2 – Demonstrate continuous H_2 production for 2 months (1440 hours) by sulfur-deprived, alginate-immobilized algae

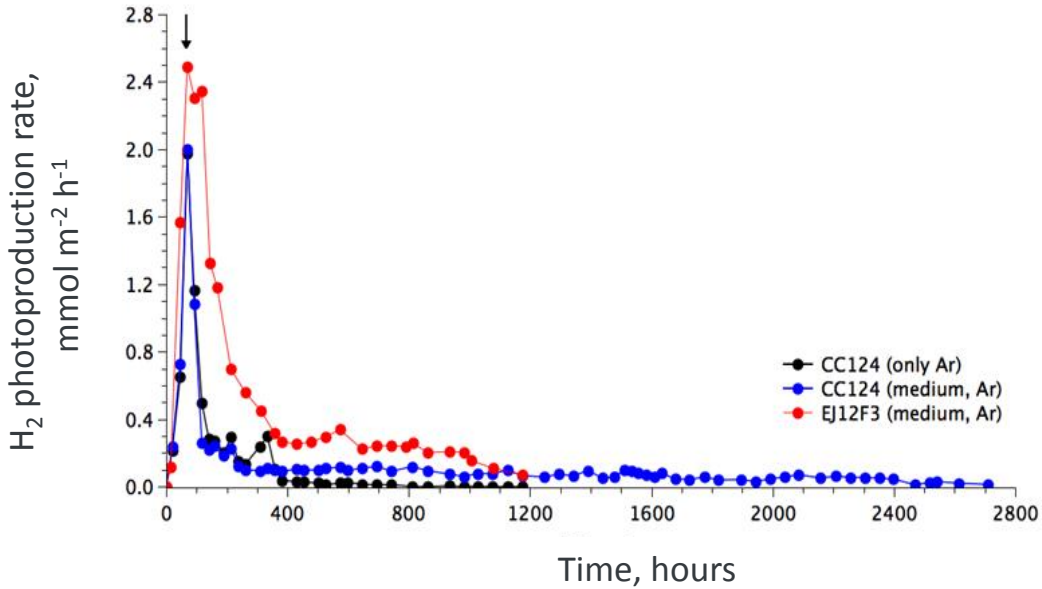
Last year's results: alginate-immobilized algae photoproduce H_2 at higher specific rates and light conversion efficiencies than cultures in suspension upon sulfur deprivation, and they show higher tolerance to aerobic environments; cycles of +S/-S resulted in prolongation of H_2 production for an additional 6 cycles of about 500 hours each.



Prolonged H_2 photoproduction was demonstrated by cycles of +S/-S

Accomplishments and Progress – Task 2

Task 2: Demonstrate continuous operation of the sulfur deprivation process for a total of 2 months (1440 hours) upon addition of phosphate/sulfate using alginate-immobilized cultures – *completed FY12 milestone 3.3.3. by 9/30/12.*



Conclusions:

- H_2 production was observed for up to 3.75 months using the wild-type CC124 strain
- The rates of production declined to very low levels after about 10 days
- A different strain of Chlamydomonas, EJ12F3 showed higher rates of H_2 photoproduction during the first 50 days.
- The economics of the process depend on the trade-off between longer H_2 production vs. lower rates

Collaborations

Partners (subcontractors) in FY12:

- Dr. Sergey Kosourov, Russian Academy of Sciences – applies sulfur deprivation to sulfur-immobilized *C. reinhardtii* cultures and tests their H_2-production capabilities (Task 2).

- Dr. Eric Johnson, Johns Hopkins University – generates ATP synthase mutants, develops transformation protocols and transforms Chlamydomonas reinhardtii; tests physiological properties of transformants (Task 2).

Summary Slide

Relevance: Photobiological systems required only water, CO_2 and minerals for cultivation; if optimized to collect additional wavelengths of light, they will have the potential of converting 17% of the solar energy into H_2 at a cost competitive with gasoline.

Approach: NREL is expressing a more O_2-tolerant bacterial hydrogenase in green algae and testing its *in vivo* tolerance to O_2.

Technical Accomplishments and Progress:

1. Successfully expressed a more O_2-tolerant, bacterial hydrogenase in a *Chlamydomonas* strain lacking native hydrogenase activity and detected more O_2-tolerant photoproduction of H_2 *in vivo*.

Collaborations: none in FY13.

Proposed Future Research: Increase the recombinant hydrogenase activity and stability in Chlamydomonas; start combining different traits to generate a more efficient H_2-producing strain of Chlamydomonas.